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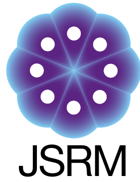

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Original Article

Multisite studies for optimization of a highly efficient culture assay used for *in vitro* detection of residual undifferentiated human pluripotent stem cells intermingled in cell therapy products

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ABSTRACT

Introduction: MEASURE2 (Multisite Evaluation Study on Analytical Methods for Non-clinical Safety Assessment of HUman-derived REgenerative Medical Products 2) is a Japanese experimental public–private partnership initiative that aims to standardize testing methods for tumorigenicity evaluation of human pluripotent stem cell (hPSC)-derived cell therapy products (CTPs). MEASURE2 organized multisite studies to optimize the methodology of the highly efficient culture (HEC) assay, a sensitive culture-based *in vitro* assay for detecting residual undifferentiated hPSCs in CTPs.
Methods: In these multisite studies, 1) the efficiency of colony formation by human induced pluripotent stem cells (hiPSCs) under two different culture conditions and 2) the sorting efficiency of microbeads conjugated to various anti-hPSC markers during hiPSC enrichment were evaluated using samples in which hiPSCs were spiked into hiPSC-derived mesenchymal stem cells.
Results: The efficiency of colony formation was significantly higher under culture conditions with the combination of Chroman 1, Emricasan, Polyamines, and Trans-ISRIB (CEPT) than with Y-27632, which is widely used for the survival of hPSCs. Between-laboratory variance was also smaller under the condition with CEPT than with Y-27632. The sorting efficiency of microbeads conjugated with the anti-Tra-1-60 antibody was sufficiently higher (>80%) than those of the other various microbeads investigated.
Conclusions: Results of these multisite studies are expected to contribute to improvements in the sensitivity and robustness of the HEC assay, as well as to the future standardization of the tumorigenicity risk assessment of hPSC-derived CTPs.
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Abbreviations: ALP, alkaline phosphatase; CEPT, Chroman 1, Emricasan, Polyamines, and Trans-ISRIB; ChiPSC18, Cellartis human iPS cell line 18; CTP, cell therapy product; CV, coefficient of variation; E8F, Essential 8 Flex; FIRM-CoNCEPT, the committee for non-clinical safety evaluation of pluripotent stem cell-derived products in the forum for innovative regenerative medicine; HEC, highly efficient culture; hiPSC, human induced pluripotent stem cell; hPSC, human pluripotent stem cell; iCell MSC, iCell mesenchymal stem cell; LOD, lower limit of detection; MACS, magnetic-activated cell sorting; MEASURE, multisite evaluation study on analytical methods for non-clinical safety assessment of human-derived regenerative medical products; ROCK, Rho-associated coiled-coil-forming protein serine/threonine kinase.

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1. Introduction

Human pluripotent stem cells (hPSCs) offer promising treatments for a wide variety of diseases for which no adequate therapy is currently available. However, hPSCs are intrinsically tumorigenic and form teratomas [1,2]; therefore, the establishment of a robust and internationally harmonized methodology to evaluate the contamination of products with residual undifferentiated hPSCs is critically beneficial, not only for product developers but also for regulatory authorities and patients [1,3]. Among the currently available *in vitro* testing methodologies [4–11] which are based on flow cytometry [4], enzyme-linked immunosorbent assay-like sandwich assay [5], quantitative RT-PCR [4,6], droplet digital PCR (ddPCR) [7–9], reverse transcription loop-mediated isothermal amplification [10], and highly efficient culture (HEC) system [11], the HEC assay is one of the most robust and sensitive assays that can directly detect hPSCs by identifying hPSC-derived colonies under culture conditions that favor their growth [11].

MEASURE (Multisite Evaluation Study on Analytical Methods for Non-clinical Safety Assessment of HUman-derived REgenerative Medical Products) is a Japanese experimental public–private partnership initiative launched by the National Institutes of Health Sciences and FIRM-CoNCEPT (the Committee for Non-Clinical Safety Evaluation of Pluripotent Stem Cell-derived ProducT, the Forum for Innovative Regenerative Medicine) with the goal of standardizing tumorigenicity-associated test methods for hPSC-derived cell therapy products (CTPs) [1]. MEASURE launched multisite studies to comprehensively evaluate the feasibility of the HEC assay and confirmed that the lower limit of detection (LOD) was 0.001% under various conditions, to which the human induced pluripotent stem cell (hiPSC) lines and culture medium/substrate were subjected [12]. In addition, the studies demonstrated that the application of magnetic-activated cell sorting (MACS) to this assay could concentrate cells expressing pluripotent stem cell markers and consequently improve detection sensitivity of the assay to a great extent (LOD = 0.00002%) [12].

As the LOD of the HEC assay is defined by the hPSC-spiked sample, the efficiency of colony formation from each spiked hPSC is highly important to ensure sensitivity of the assay. Additionally, insufficient colony formation efficiency may cause false-negative results when hPSC-derived CTPs are evaluated; therefore, securing a higher colony formation rate is essential for this assay. Recently, Chen et al. [13] reported that the combination of Chroman 1, Emricasan, Polyamines, and Trans-ISRIB (CEPT) enhanced the survival of hPSCs, and its cytoprotective effects were superior to those of Y-27632, a Rho-associated coiled-coil-forming protein serine/threonine kinase (ROCK) inhibitor, which has been widely used to improve the survival of hPSCs. Therefore, MEASURE2, a subsequent project of MEASURE, organized this multisite study to compare the colony formation efficiency of hiPSCs spiked into hiPSC-derived mesenchymal stem cells (MSCs) between culture conditions wherein CEPT and Y-27632 were used. In addition, MEASURE2 conducted this multisite study to evaluate the sorting efficiency of microbeads conjugated to various anti-pluripotent stem cell markers to optimize the MACS process.

2. Material and methods

2.1. Cells

hiPSCs: The hiPSC lines, Cellartis human iPS cell line 18 (ChiPSC18) and 201B7, were used in this study. ChiPSC18 cells were purchased from Takara Bio Inc. (Shiga, Japan), and 201B7 cells obtained from RIKEN BRC (Kyoto, Japan). ChiPSC18 cells were maintained using the Cellartis DEF-CS Culture System (Takara Bio Inc.), according to the manufacturer's instructions. When passaged every 3–7 days, cells were detached by treatment with 1 × TrypLE Express (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 5–7 min at 37 °C and re-seeded at 1.5–5 × 10^4 cells/cm^2 on culture dishes coated with DEF-CS COAT-1 in DEF-CS medium. 201B7 cells were maintained in the defined culture medium, StemFit AK02N or StemFit AK03N (Ajinomoto Co., Inc., Tokyo, Japan), in accordance with a previously reported procedure [14], with minor modifications. Every 3–7 days at 80% confluency, cells were passaged through treatment with a detachment solution consisting of 0.5 × TrypLE Express (1 × TrypLE Express diluted with 0.5 mM EDTA/phosphate-buffered saline [Nacalai Tesque Inc., Kyoto, Japan, or Thermo Fisher Scientific Inc.] to a final concentration of 0.75 mM EDTA) for up to 7 min at 37 °C. Cells were seeded at 0.2–2 × 10^4 cells/cm^2 onto culture dishes in StemFit AK02N or AK03N medium supplemented with 10 μM of the ROCK inhibitor, Y-27632 (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan), and 0.25 μg/cm^2 iMatrix-511 solution (Nippi Inc., Tokyo, Japan). Following a 24-h recovery, the cells were fed StemFit AK02N or AK03N medium alone for the remainder of the culture period. The culture medium volume applied to culture dishes was adjusted to 200 μL/cm^2. The frozen hiPSCs, ChiPSC18 (passages 20–22), and 201B7 (passages 24–44) were thawed and used for experiments after 2–8 passages.

iCell MSCs: For the hiPSC-derived MSCs, iCell MSCs (#C1043) were purchased from FUJIFILM Cellular Dynamics, Inc. (Madison, WI, USA) and cultured in MSCGM medium (Lonza Inc., Basel, Switzerland). The iCell MSCs from passage 2 were used in this study.

2.2. Expression profile of pluripotent stem cell markers

Wash and staining buffers were freshly prepared before sample preparation by diluting MACS BSA Stock Solution (#130-091-376; Miltenyi Biotec, Bergisch Gladbach, Germany) in autoMACS Rinsing Solution (#130-091-222; Miltenyi Biotec) at a ratio of 1:20. ChiPSC18 and 201B7 cells were dissociated into a single-cell suspension, using the respective methods described in Section 2.1, and thereafter fixed with 4% paraformaldehyde and washed with buffer. Subsequently, the cells were incubated with either PE-conjugated anti-human SSEA-4 (1:50; #130-122-958; Miltenyi Biotec), anti-human Tra-1-60 (1:50; #130-122-965; Miltenyi Biotec), anti-human CD326 (1:50; #130-111-116; Miltenyi Biotec), or recombinant anti-human IgG1 control (1:50; #130-113-438; Miltenyi Biotec) antibodies at 4 °C for 30 min. Then, all samples were washed twice with buffer, processed through a CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA, USA), and analyzed on CytExpert 2.4 or Kaluza Analysis 2.1 software (Beckman Coulter).

2.3. HEC assay

The iCell MSCs were suspended in Essential 8 Flex (E8F) medium (Thermo Fisher Scientific) containing 50 nM Chroman 1 (#HY-15392; MedChem Express, Monmouth Junction, NJ, USA), 5 μM Emricasan (#S7775; Selleckchem, Houston, TX, USA), Polyamine supplement (#P8483, 1:1000; Sigma-Aldrich, St. Louis, MO, USA), and 0.7 μM Trans-ISRIB (#5284; Tocris Bioscience, Bristol, UK) (E8F+CEPT [12] medium), or 10 μM Y-27632 (E8F+Y medium). These cells were seeded onto the wells of 6-well plates coated with laminin-521 (BioLamina AB, Sundbyberg, Sweden) [11] at a cell density of 1 × 10^5 cells per well. The hiPSCs, ChiPSC18, or 201B7 cells were dissociated into single cells using TrypLE Express. After confirming cell viability (>ca. 80%) using a Countess automated cell counter (settings: sensitivity, 5 min; size, 8; maximum size: 30; circularity, 75), a TC20 automated cell counter (settings: minimum size, 8; maximum size, 30), or manually with a counting chamber,

the cells were suspended in E8F medium, and 5×10^1 cells/mL of the ChiPSC18 or 201B7 cell suspension was carefully prepared via serial dilution from 5×10^5 cells/mL of each cell suspension at a ratio of 1:10. Immediately after dilution, 0.1 or 0.2 mL of a 5×10^1 cells/mL suspension of ChiPSC18 or 201B7 was added to the wells previously seeded with iCell MSCs suspended in each E8F+Y or E8F+CEPT media to spike 5 (0.005%) or 10 (0.01%) hiPSCs into 1×10^5 iCell MSCs. One (for the E8F+Y medium) or 3 (for the E8F+CEPT medium) days after seeding, the media were replaced by E8F medium, and after 6 or 7 days of incubation at 37 °C in 5% CO_2, the wells were fixed and stained using the VECTOR Blue Alkaline Phosphatase (ALP) Substrate Kit (VECTOR Laboratories Inc., Newark, CA, USA), according to a previously reported method [12]. ALP-positive hiPSC colonies were counted manually under a microscope by two different operators, and the colony formation rate, which is the ratio of the total number of colonies to the number of spiked hiPSCs, was calculated. These assays were conducted at five facilities (A, B, C, D, and E). Each assay was performed using five wells per condition and repeated three times at each facility.

2.4. MACS

The sorting efficiency of spiked hiPSCs via the MACS system was investigated using microbeads conjugated to several anti-pluripotent stem cell markers, including Tra-1-60, SSEA-4, and CD326. The hiPSCs, ChiPSC18, or 201B7 cells were suspended in E8F+CEPT medium, and 5×10^2 cells/mL of the ChiPSC18 or 201B7 cell suspension was carefully prepared in the same manner as previously described in Section 2.3. Immediately after the dilution, 0.1 mL cell suspensions of 5×10^2 cells/mL ChiPSC18 or 201B7 was mixed with cell suspensions of iCell MSCs previously suspended in E8F+CEPT medium to prepare suspensions containing 50 hiPSCs (0.005%) in 1×10^6 iCell MSCs. The cell suspension was spun down at 300×g for 5 min at room temperature, and after the supernatant was discarded, the cell pellets were reconstituted in 80 μL E8F+CEPT medium. Then, the cells were incubated with either 20 μL anti-human Tra-1-60 (#130-100-832; Miltenyi Biotec), anti-human SSEA-4 (#130-097-855; Miltenyi Biotec), or anti-human CD326 microbeads (Pluripotent Stem Cell Microbeads, #130-095-804; Miltenyi Biotec) at 4 °C for 15 min. Furthermore, the cells reconstituted in 40 μL E8F+CEPT medium after centrifugation were incubated with the mixture of 20 μL anti-human Tra-1-60, 20 μL anti-human SSEA-4, and 20 μL anti-human CD326 microbeads at 4 °C for 15 min. After incubation with the microbeads, the suspensions were reconstituted in 1 mL E8F+CEPT medium and applied to an MS column attached to a MidiMACS separator (Miltenyi Biotec). After the column was washed with E8F+CEPT medium, the cells retained inside the column were gently flushed out with 1 mL E8F+CEPT medium, and the eluted suspensions seeded onto the wells of 6-well plates coated with laminin-521. Cell suspensions containing 50 or no hiPSCs, ChiPSC18, or 201B7 cells in 1×10^5 iCell MSCs were prepared and seeded onto the wells of 6-well plates in the same manner without MACS. Three days after seeding, the medium was replaced by E8F medium. After 7 days of incubation, ALP-positive hiPSC colonies were counted manually under a microscope, and the sorting ratio (the ratio of the number of colonies from 50 spiked hiPSCs after MACS with each microbead condition to that without MACS) was calculated. These assays were conducted at five facilities (A, B, C, D, and E). Each assay was performed in duplicate and repeated three times at each facility.

2.5. Statistical analysis

For the experiment with CEPT medium, the number of hiPSC colonies and the colony formation rate were statistically analyzed via four-way analysis of variance (ANOVA), followed by the Bonferroni method, using Excel Statistics ver. 7.0 (Esumi Co., Ltd., Tokyo, Japan). For the sorting efficiency of MACS pretreatment, the sorting ratio was statistically analyzed via three-way ANOVA, followed by the Bonferroni method. Statistical significance was set at $p < 0.05$.

The repeatability and reproducibility of the colony formation rate were statistically analyzed for the experiment with CEPT medium, according to the formula described in ISO 5725 [15,16], where the reproducibility variance is defined as the sum of the repeatability (intra-laboratory variance) and between-laboratory variances.

Variability from a multisite study can be modeled as:

$$s_R^2 = s_L^2 + s_r^2$$

where s_R^2 is the reproducibility variance, s_L^2 the between-laboratory variance, and s_r^2 the repeatability variance.

The value of s_r^2 is calculated by:

$$s_r^2 = \frac{\sum_{i=1}^{p}(n_i - 1)s_i^2}{\sum_{i=1}^{p}(n_i - 1)}$$

where p is the number of laboratories, n_i the number of test results in the i-th laboratory, and s_i the standard deviation of the test results in the i-th laboratory.

The value of s_L^2 is calculated by:

$$s_L^2 = \begin{cases} s_m^2 - s_r^2 \big/ \hat{n}, & s_m^2 > s_r^2 \big/ \hat{n} \\ 0, & s_m^2 \le s_r^2 \big/ \hat{n} \end{cases}$$

where

$$s_m^2 = \frac{1}{p-1} \cdot \frac{\sum_{i=1}^{p} n_i(\bar{y}_i - \hat{m})^2}{\hat{n}}$$

$$\hat{n} = \frac{1}{p-1}\left[\sum_{i=1}^{p} n_i - \frac{\sum_{i=1}^{p} n_i^2}{\sum_{i=1}^{p} n_i}\right]$$

$$\hat{m} = \frac{\sum_{i=1}^{p} n_i \bar{y}_i}{\sum_{i=1}^{p} n_i}$$

and $\bar{y}_i$ is the mean of results in the i-th laboratory.

3. Results

3.1. Comparison of the colony formation efficiency of hiPSCs between CEPT and Y-27632 treatment

The mean number of ALP-positive hiPSC colonies and the colony formation rate (the ratio of the total number of colonies to the number of spiked hiPSCs) from three experiments of each hiPSC line or culture condition are shown in Tables 1 and 2 and Fig. 1.

The hiPSC colonies were confirmed in both the 5 and 10 hiPSC-spiked conditions for both hiPSC lines (ChiPSC18 and 201B7) under each culture condition (CEPT or Y-27632) at all five facilities

Table 1
Number of alkaline phosphatase-positive human induced pluripotent stem cell colonies in the highly efficient culture assay.

ChiPSC18##				
Culture condition##	CEPT		Y-27632	
Number of spiked hiPSCs##	5	10	5	10
Facility A[e]	4.0 ± 2.2	6.8 ± 1.7	2.0 ± 0.7	5.5 ± 2.7
Facility B	2.8 ± 0.9	6.5 ± 1.7	1.8 ± 0.9	4.4 ± 1.2
Facility C[e]	3.5 ± 0.4	7.6 ± 0.5	2.6 ± 0.3	6.3 ± 0.6
Facility D[e]	4.0 ± 0.7	5.2 ± 2.3	3.6 ± 0.8	5.1 ± 1.9
Facility E[a,c,d]	2.6 ± 0.4	5.2 ± 1.6	0.9 ± 0.6	3.1 ± 0.3
201B7##				
Culture condition##	CEPT		Y-27632	
Number of spiked hiPSCs##	5	10	5	10
Facility A[b,d,e]	2.6 ± 0.9	7.3 ± 2.4	2.3 ± 0.4	3.9 ± 0.3
Facility B[a,c,e]	2.2 ± 1.2	4.5 ± 2.0	1.0 ± 0.8	2.3 ± 1.5
Facility C[b,d,e]	3.7 ± 0.2	7.7 ± 0.1	2.3 ± 0.1	6.1 ± 0.3
Facility D[a,c]	2.3 ± 1.1	3.3 ± 0.8	1.1 ± 0.2	2.0 ± 0.8
Facility E[a,b,c]	1.1 ± 0.6	2.3 ± 0.8	0.3 ± 0.1	0.7 ± 0.4

All values (mean ± standard deviation) were obtained from three quintuplicate repeats.
Four-way analysis of variance (ANOVA) indicated that there were significant differences ($p < 0.01$) in the "Number of spiked hiPSCs" (5 vs. 10), "Culture condition" (CEPT vs. Y-27632), "hiPSC line" (ChiPSC18 vs. 201B7), and "Facility" (A–E), as well as interactions between the "Facility" and "hiPSC line" and between the "Facility" and "Number of spiked hiPSCs".
[a, b, c, d, e] indicate significant differences ($p < 0.05$) observed from facilities A–E, respectively, as calculated using Bonferroni's post-hoc multiple comparison test. See Supplemental Table S1 for other results of the post-hoc multiple comparison tests.
CEPT: Chroman 1, Emricasan, Polyamines, and Trans-ISRIB; hiPSC: human induced pluripotent stem cell.

(Table 1). The number of hiPSC colonies at each 5 and 10 hiPSC-spiked condition was significantly different according to the four-way ANOVA results. A significant interaction was observed between the "Facility" and "Number of spiked hiPSCs"; however, significant differences between the numbers of spiked iPSCs were confirmed across all facilities via Bonferroni's post-hoc multiple comparison test [Supplementary Table S1 (E)], indicating that differences in the number of hiPSC colonies between the 5 and 10 hiPSC-spiked conditions could be clearly distinguished. Both the number of hiPSC colonies and colony formation rates were significantly higher under the CEPT condition than under the Y-27632 condition (**$p < 0.001$), and no significant interaction was observed between the other factors (number of spiked hiPSCs, hiPSC line, and facility) (Tables 1 and 2). When comparing the two hiPSC lines, the number of hiPSC colonies and the colony formation rate of ChiPSC18 showed significantly higher values compared to those of 201B7. In addition, significant interactions were observed between the "Facility" and "hiPSC line" (Tables 1 and 2). The number of hiPSC colonies and colony formation rates also showed significant differences among the facilities, possibly due to the lower values observed in facility E. Bonferroni's post-hoc multiple comparison test revealed that significant differences between hiPSC lines were not always observed in all facilities [Supplementary Table S1 (F and G) and S2 (D and E)]. Therefore, it is likely that the significant differences observed between the two hiPSC lines were mainly influenced by differences among the facilities.

Table 2
Colony formation rate (%) in the highly efficient culture assay.

ChiPSC18##				
Culture condition##	CEPT		Y-27632	
Number of spiked hiPSCs	5	10	5	10
Facility A[e]	80 ± 44	68 ± 17	40 ± 13	55 ± 27
Facility B	56 ± 18	65 ± 17	36 ± 18	44 ± 12
Facility C[e]	69 ± 8	76 ± 5	51 ± 5	63 ± 6
Facility D[e]	81 ± 14	52 ± 23	71 ± 16	51 ± 19
Facility E[a,c,d]	52 ± 8	52 ± 16	19 ± 12	31 ± 3
201B7##				
Culture condition##	CEPT		Y-27632	
Number of spiked hiPSCs	5	10	5	10
Facility A[b,d,e]	51 ± 17	73 ± 24	47 ± 8	39 ± 3
Facility B[a,c,e]	44 ± 25	45 ± 20	20 ± 16	23 ± 15
Facility C[b,d,e]	73 ± 5	77 ± 1	46 ± 2	61 ± 3
Facility D[a,c]	45 ± 21	33 ± 8	22 ± 3	20 ± 8
Facility E[a,b,c]	23 ± 12	23 ± 8	5 ± 2	7 ± 4

All values (mean ± standard deviation) were obtained from three quintuplicate repeats.
Four-way analysis of variance (ANOVA) indicated that there were significant differences ($p < 0.01$) in the "Culture condition" (CEPT vs. Y-27632), "hiPSC line" (ChiPSC18 vs. 201B7), and "Facility" (A–E), as well as interactions between the "Facility" and "hiPSC line".
[a, b, c, d, e] indicate significant differences ($p < 0.05$) observed from facilities A–E, respectively, as calculated using Bonferroni's post-hoc multiple comparison test. See Supplemental Table S2 for other results of the post-hoc multiple comparison tests.
CEPT: Chroman 1, Emricasan, Polyamines, and Trans-ISRIB; hiPSC: human induced pluripotent stem cell.

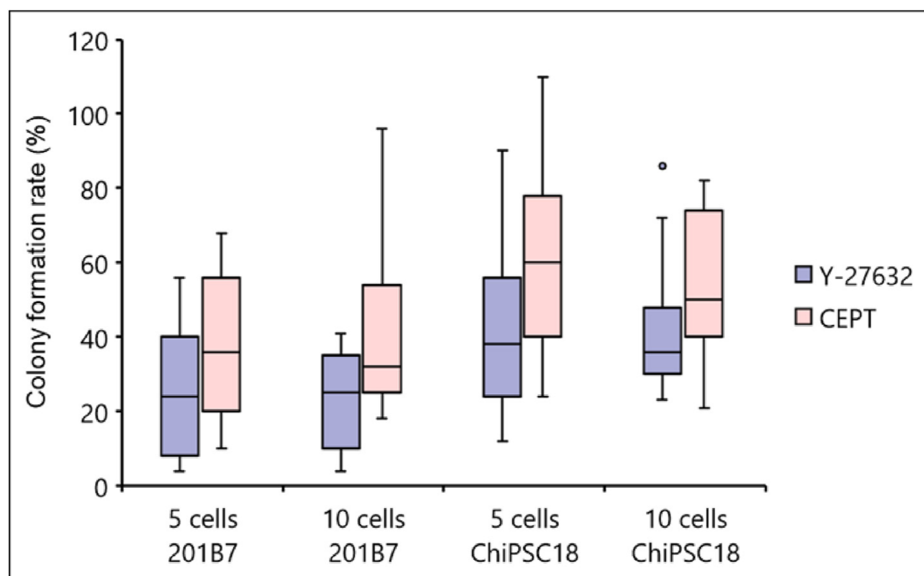

Fig. 1. Colony formation rate. Colony formation rates measured under various conditions are indicated by the box-and-whisker plots. All data represent the results of three quintuplicate experiments conducted in five different facilities under specific conditions. Median values are marked by horizontal lines. Boxes indicate 25–75% data ranges, and the range outside 1.5 times the interquartile range is shown as whiskers. The outliers are plotted as open circles.

The general mean colony formation rate tended to differ between the hiPSC lines and between the culture conditions, which appeared to be consistent with the data shown in Table 2, but was not significantly different, likely because each comparison in the three-way ANOVA had only one degree of freedom. The standard deviations of repeatability (S_r) and reproducibility (S_R) of the colony formation rate were not dependent on the three test factors: hiPSC line, culture condition, and number of spiked hiPSCs. The coefficients of variation (CVs) for repeatability [CV (S_r)] and for reproducibility [CV (S_R)] ranged from 26 to 37% and from 27 to 72%, respectively. The CV (S_R) was significantly different between the hiPSC lines and tended to be lower in the CEPT condition than in the Y-27632 condition ($p = 0.07$) (Table 3).

3.2. Sorting efficiency of microbeads conjugated to various anti-pluripotent stem cell markers

Before evaluating the sorting efficiency of the microbeads used to concentrate cells expressing pluripotent stem cell markers, we investigated the expression profile of pluripotent stem cell markers in the hiPSC lines used in the present study (ChiPSC18 and 201B7) via flow cytometric analysis. Tra-1-60, SSEA-4, and CD326 were selected as markers because microbeads that could conjugate to them were commercially available. The expression profiles of Tra-1-60, SSEA-4, and CD326 were almost similar in these two hiPSC lines; the percentage of SSEA-4- and CD326-positive cells was almost 100%, and that of Tra-1-60-positive cells slightly lower at approximately 85–90% (Fig. 2).

The mean numbers of ALP-positive hiPSC colonies with or without MACS treatment using microbeads conjugated to anti-Tra-1-60, anti-SSEA-4, and anti-CD326 are shown in Supplementary Table S3. The sorting ratio, which is the ratio of the number of colonies after MACS with each microbead condition to that without MACS, is shown in Table 4 and Fig. 3. Three-way ANOVA performed on the sorting ratio revealed significant differences among the different microbead conditions (**$p < 0.001$), and significant interactions were also noted between the “Facility” and “Microbeads” and among the “Facility,” “Microbeads,” and “hiPSC line.” Anti-Tra-1-60 microbeads led to the highest sorting ratio, although this was not statistically different from that of anti-CD326 microbeads or a mixture of the three microbead types (Fig. 3). In contrast, the sorting ratio with anti-SSEA-4 microbeads was significantly lower than that with other conditions for both cell lines [Supplementary Table S4 (A)]. In addition, Bonferroni's post-hoc multiple comparison test confirmed that significantly lower values under the condition of anti-SSEA-4 microbeads were frequently observed in all

Table 3
Statistical analysis results of the repeatability and reproducibility of the colony formation rate (%) in the highly efficient culture assay.

hiPSC line	ChiPSC18				201B7			
Culture condition	CEPT		Y-27632		CEPT		Y-27632	
Number of spiked hiPSCs	5	10	5	10	5	10	5	10
General mean of colony formation rate, $\hat{m}$[a]	67.6	62.9	43.5	48.9	47.3	50.1	28.0	30.1
Repeatability standard deviation, s_r	22.7	16.4	13.7	16.0	17.6	14.8	8.3	8.2
Reproducibility standard deviation, s_R	22.8	17.1	22.5	18.0	23.1	26.8	19.2	21.7
Repeatability, CV (s_r)	34%	26%	31%	33%	37%	29%	30%	27%
Reproducibility, CV (s_R)[b]	34%	27%	52%	37%	49%	54%	68%	72%

The p-values of the other comparisons for the three factors in the five parameters were greater than 0.14 [three-way analysis of variance (ANOVA) with one degree of freedom for each comparison].
CEPT: Chroman 1, Emricasan, Polyamines, and Trans-ISRIB; CV: coefficient of variation; hiPSC: human induced pluripotent stem cell.
[a] $p = 0.10$ between hiPSC lines and $p = 0.09$ between culture conditions.
[b] $p < 0.05$ between hiPSC lines and $p = 0.07$ between culture conditions.

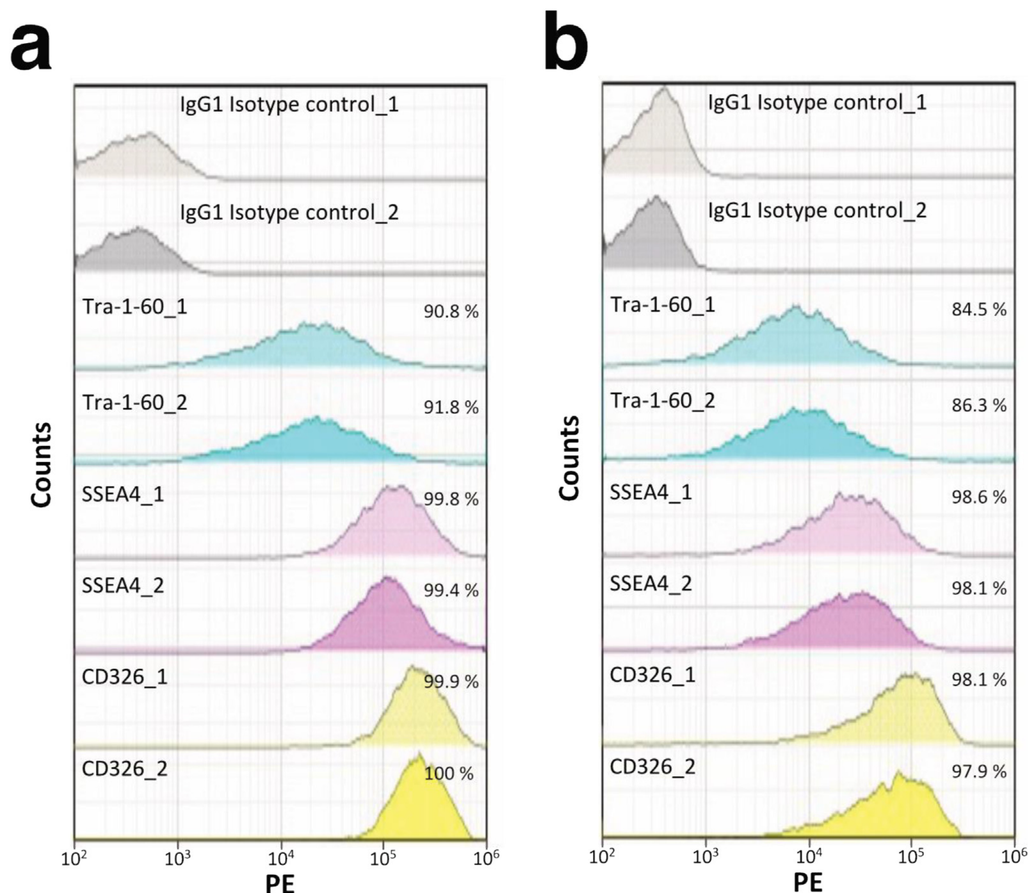

Fig. 2. Expression profile of Tra-1-60, SSEA-4, and CD326 in the ChiPSC18 and 201B7 cell lines. Representative flow cytometry diagrams showing Tra-1-60, SSEA-4, and CD326 expression in the ChiPSC18 (a) and 201B7 (b) cell lines; the percentage of cells in the positive gate is indicated.

Table 4
Sorting ratio after magnetic-activated cell sorting using microbeads conjugated to anti-human pluripotent stem cell markers.

ChiPSC18##				
	Microbeads##			
	Tra-1-60[b]	SSEA-4[a,c,d]	CD326[b]	Tra/SSEA/CD[b]
Facility A	66 ± 18	26 ± 7	51 ± 3	67 ± 5
Facility B	90 ± 53	40 ± 5	51 ± 15	69 ± 10
Facility C	87 ± 23	25 ± 11	74 ± 27	71 ± 10
Facility D	87 ± 9	26 ± 13	85 ± 12	111 ± 17
Facility E	73 ± 1	54 ± 8	77 ± 14	72 ± 13
201B7##				
	Microbeads##			
	Tra-1-60[b]	SSEA-4[a,c,d]	CD326[b]	Tra/SSEA/CD[b]
Facility A	80 ± 19	62 ± 12	82 ± 21	80 ± 21
Facility B	89 ± 1	35 ± 8	68 ± 30	89 ± 8
Facility C	90 ± 9	92 ± 15	77 ± 14	58 ± 16
Facility D	88 ± 20	43 ± 4	98 ± 23	90 ± 23
Facility E	88 ± 14	34 ± 8	68 ± 20	78 ± 19

Sorting ratio: ratio of the number of colonies after magnetic-activated cell sorting (MACS) with each microbead condition to that without MACS. All data represent the mean ± standard deviation obtained from three duplicate experiments at each facility. Tra/SSEA/CD: mixture of anti-human Tra-1-60, anti-SSEA-4, and anti-CD326 microbeads.

##Three-way analysis of variance (ANOVA) indicated that there were significant differences ($p < 0.01$) in the "Microbeads" (Tra-1-60 vs. SSEA-4 vs. CD326 vs. Tra/SSEA/CD) and "hiPSC line" (ChiPSC18 vs. 201B7), as well as interactions between the "Facility" and "Microbeads" ($p < 0.05$) and among the "Facility", "Microbeads", and "hiPSC line" ($p < 0.05$).

[a, b, c, d] indicate significant differences ($p < 0.05$) from Tra-1-60, SSEA-4, CD326, and Tra/SSEA/CD, respectively, as calculated using Bonferroni's post-hoc multiple comparison test. See Supplemental Table S4 for other results of the post-hoc multiple comparison tests.

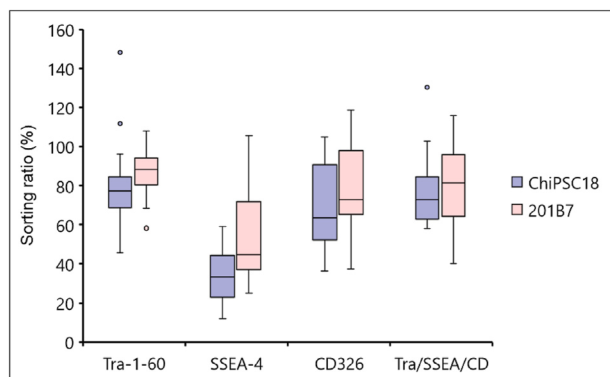

Fig. 3. Sorting ratio. Sorting ratios measured under various conditions are indicated by box-and-whisker plots. All data represent the results of three duplicate experiments conducted in five different facilities under specific conditions. Median values are marked by horizontal lines [(Tra-1-60:SSEA-4:CD326:Tra/SSEA4/CD); 80.6%:34.0%:67.4%:78.0% for ChiPSC18 and 87.1%:53.2%:78.5%:79.3% for 201B7]. Boxes indicate 25–75% data ranges, and the range outside 1.5 times the interquartile range is shown as whiskers. The outliers are plotted as open circles. Tra/SSEA/CD: mixture of anti-human Tra-1-60, SSEA-4, and CD326 microbeads.

facilities [Supplementary Table S4 (C)]. The sorting ratio of 201B7 cells was significantly higher than that of ChiPSC18 cells [Supplementary Table S4 (B)]. For non-spiked samples, no colonies were observed in any wells at any of the facilities.

4. Discussion

The HEC assay is a simple culture-based assay that can directly detect hPSCs by identifying hPSC-derived colonies under culture conditions that favor their growth [11]. As the readout of the assay identifies hPSC-derived colonies, securing a high colony formation rate is, therefore, essential.

It is well known that enzymatic dissociation of hPSCs causes cell death via apoptosis and anoikis [17–19]. After the identification of Y-27632, a selective inhibitor of p160-ROCK [20,21], it has been widely used in various applications in stem cell research [22] as a factor that enhances hPSC survival upon single-cell dissociation [23]. In a previous MEASURE multisite study, after culture medium was supplemented with Y-27632, all facilities could detect hiPSC-derived colonies under the condition that 10 hiPSCs were spiked into one million primary human mesenchymal stromal cells, regardless of the conditions under which the hiPSC lines and culture medium/substrate were subjected (LOD = 0.001%) [12]. However, the mean number of hiPSC-derived colonies from three duplicate experiments at some facilities was lower than 1.0 under a few experimental conditions of hiPSC lines and culture medium/substrate, indicating that improvement of culture conditions is desirable to enhance the sensitivity and robustness of the assay.

Recently, CEPT, a small-molecule cocktail comprising Chroman 1, Emricasan, Polyamines, and Trans-ISRIB, was reported to have superior cytoprotective effects in hPSCs when compared to Y-27632 [13]. In addition, a recent international evaluation of the HEC assay indicated that CEPT had more beneficial effects on colony formation efficiency in the HEC assay than Y-27632 did [24]. However, this study did not evaluate colony formation affected by CEPT and Y-27632 on a head-to-head basis; therefore, in the present MEASURE2 study, a direct comparison was made between the number of hiPSC-derived colonies under the culture conditions supplemented with Y-27632 or CEPT when hiPSCs were spiked into hiPSC-derived MSCs. As a result, the hiPSC colonies could be detected even in the 5 hiPSC-spiked conditions of both ChiPSC18 and 201B7 hiPSCs under both culture conditions at all five facilities. However, the mean number of hiPSC colonies was lower than 1.0 at facility E for both hiPSC lines when cultured with Y-27632, whereas that when cultured under CEPT conditions was larger than 1.0 at all facilities. Statistical analysis revealed that the efficiency of hiPSC colony formation under CEPT conditions was significantly higher than that under Y-27632 conditions. Importantly, there were no significant interactions between the other factors investigated (number of spiked hiPSCs, hiPSC lines, and facilities). Thus, the higher colony formation efficiency observed under CEPT than under Y-27632 conditions could be robustly observed, regardless of the factors that could influence the formation of hiPSC colonies. In addition, analysis of the repeatability and reproducibility of the colony formation rate showed that the CV values of reproducibility tended to be lower under CEPT conditions compared to those under Y-27632 conditions (CEPT: 27–54%, Y-27632: 37–72%), although the CV values of repeatability were almost similar between the two culture conditions (CEPT: 26–37%, Y-27632: 27–33%). As the reproducibility variance is the sum of the repeatability (intra-laboratory variance) and between-laboratory variances, it can be noted that CEPT has the potential to reduce between-laboratory variance. Non-hiPSC-spiked samples were not prepared in these experiments; however, there was no colony formation in hiPSC-derived MSCs cultured with CEPT in the experiments to evaluate the sorting efficiency of MACS, indicating that there was no contamination of hiPSC-derived MSCs with undifferentiated hiPSCs. Overall, these results indicate that colony formation efficiency under CEPT conditions is superior to that under Y-27632 conditions and, therefore, CEPT could serve as an efficient tool to enhance the sensitivity and robustness of the HEC assay. However, these experiments were performed using hiPSC-derived MSCs, which are adherent cells, and not hiPSC-derived suspension cells; thus, further experiments spiking iPSCs into cell suspensions are needed.

In the present multisite study, the colony formation efficiency showed significant differences between the two hiPSC lines and among the facilities. A significant interaction was also observed between hiPSC lines and facilities, and the multiple comparison test indicated that the differences observed in colony formation efficiency between hiPSC lines mainly resulted from differences among the facilities. This indicates that practical training, especially on the accurate execution of both the preparation of hiPSC cell suspensions and spiking of these cells into the samples, might reduce the differences observed between hiPSC lines and among the facilities in this study. However, there is a possibility that colony formation could be influenced by the factors of hiPSC cell lines and facilities. For instance, regarding the differences between hiPSC lines, the difference in culture conditions between the HEC assay and those required for the maintenance of hiPSCs may have some impact on the colony formation efficiency and this effect was different between the hiPSC lines. Thus, it is important to define the LOD of the assay for each CTP at each test facility through a spiking experiment with the hPSC line, which is used as a raw material for the product. Additionally, potential factors of the product side on the variation in the colony formation efficiency should be considered, especially when there are some cellular populations of the product producing the factors which affect the survival of undifferentiated hPSCs.

A previous MEASURE multisite study demonstrated that detection sensitivity of the HEC assay could be drastically improved by adding a step consisting of enriching the targeted undifferentiated hPSCs via a MACS system [12]. In the present MEASURE2 multisite study, the sorting efficiency of microbeads conjugated to various anti-pluripotent stem cell markers was evaluated to optimize the MACS process. Two hiPSC lines, ChiPSC18 and 201B7, were used, and the pluripotent stem cell markers, Tra-1-60, SSEA-4, and CD326, were selected based on the commercial availability of

microbeads that could be conjugated to the anti-markers. The anti-Tra-1-60 and anti-SSEA-4 antibodies were reported to recognize keratan-sulfated proteoglycan [25] and NeuAcα2-3Galβ1-3GalNAc [26], respectively, both of which are expressed on the membrane surface of hPSCs. CD326, also termed the epithelial cell adhesion molecule, is a transmembrane glycoprotein that was identified as a surface marker on undifferentiated hPSCs [27]. In fact, high expression of these selected markers was confirmed in both hiPSC lines through flow cytometric analysis. The percentage of SSEA-4- and CD326-positive cells was almost 100%, whereas that of Tra-1-60-positive cells was approximately 85–90%. Unexpectedly, the sorting efficiency using anti-SSEA-4 microbeads was significantly lower than that when using anti-Tra-1-60 or anti-CD326 microbeads for both ChiPSC18 and 201B7 cells. The reason why the sorting efficiency was lower when using anti-SSEA-4 microbeads, despite there being a higher expression of SSEA-4 on both hiPSC lines, remains to be elucidated. It is possible that binding of the anti-SSEA-4 antibody to hiPSCs might impair their survival and proliferation, and that the dissociation kinetics from the antigen could be different between the two antibodies. In addition, each microbead was treated according to the manufacturer's instructions, and further optimization of the process may be required for the application of anti-SSEA-4 microbeads in the HEC assay. For the other microbeads (anti-Tra-1-60 and anti-CD326), the sorting efficiencies were not significantly different, although this was slightly higher in the presence of anti-Tra-1-60 for both hiPSC lines, even though its expression was slightly lower than that of SSEA-4 and CD326. The condition using a mixture of three microbeads was also investigated in this study; however, this mixture did not show any additive effects on sorting efficacy, possibly because of the already higher sorting efficiencies of the anti-Tra-1-60 and CD326 microbeads. Thus, among the conditions investigated in this multisite study, anti-Tra-1-60 microbeads were considered the most suitable for enriching the targeted undifferentiated hPSCs. However, considering the discrepancy between the expression profile and sorting efficiency, and the significant differences in the sorting efficiency observed between the two hiPSC lines in this study, it is worth noting that the marker used for MACS should be carefully validated using the hPSC line, which will be spiked for the HEC assay, regardless of the expression profile of the marker on the hPSC line. In the MEASURE2 study, the LOD using each microbead was not confirmed; however, in the previous MEASURE study, 10 hiPSCs spiked into 5×10^7 human T cells could be detected in the HEC assay in the presence of Y-27632 after MACS using anti-human TRA-1-60 microbeads (LOD = 0.00002%) [12].

In conclusion, the MEASURE2 multisite study using hiPSC-derived MSCs proved that culture conditions with CEPT are more efficient for the colony formation of hiPSCs compared to those with Y-27632, which has been widely used for the HEC assay. It was also confirmed that the sorting efficiency of microbeads conjugated to the anti-Tra-1-60 antibody was the highest (>80%) among the various microbeads investigated, and that the MACS process with such a validated microbead is an effective tool for enhancing sensitivity of the HEC assay by enriching the targeted undifferentiated hPSCs. Overall, these findings indicate that these assay optimization steps are valuable for further improvement of the sensitivity and robustness of the HEC assay and could also contribute to the future standardization of tumorigenicity risk assessment of hPSC-derived CTPs to ensure their safety and quality.

Author contributions

T.W., S.Y., and Y.S. conceived and designed the experiments. S.K, T.K., H.F., M.F., S.S., A.M., S.H., A.K., and K.K. performed the experiments. T.W., S.Y., and Y.S. wrote the manuscript. S.Y. and Y.S. acquired funding. All authors analyzed the data and reviewed the manuscript.

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Data availability statement

Not applicable.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.06.007.

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